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# Quantification of regional glial fibrillary acidic protein levels in Alzheimer's disease

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*Objectives* – Our objectives were to quantify glial fibrillary acidic protein (GFAP) in brains of Alzheimer's disease (AD) cases, and non-AD controls to determine the regions with the most severe gliosis in AD. *Material and methods* – In a case–control design, we used an enzyme-linked immunosorbent assay (ELISA) to quantify GFAP in frozen brain from four areas of neocortex in 10 AD cases, 10 age-matched controls, and 10 younger controls from the Honolulu-Asia Aging Study autopsy archive. *Results* – Median age at death was 83.5 years for cases and age-matched controls, and 77 years for younger controls. For the AD cases compared with the age-matched controls, levels of GFAP in occipital ($P = 0.01$), parietal ($P = 0.028$), and temporal lobes ($P = 0.004$) (but not frontal) were significantly higher in the cases. The median GFAP excess in AD cases compared with age matched controls was highest in the temporal lobe. *Conclusions* – Regional quantification of GFAP reveals that the glial response is most prominent in the temporal lobe in AD.

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Astrogliosis, characterized by enhanced expression of the major intermediate filament protein of astrocytes, glial fibrillary acidic protein (GFAP), is a homotypic response to brain injury. In animal models, the degree and duration of neural damage is reflected by the degree and duration of astrogliosis (1, 2). Often, damage-induced elevations in GFAP as assessed by enzyme-linked immunosorbent assay (ELISA) can be observed in the absence of overt cytopathology (2). Thus, increases in GFAP serve as a sensitive and quantitative index of neural damage.

The presence of neuritic senile plaques (SP), one of the neuropathologic hallmarks of Alzheimer's disease (AD), is associated with immunohistochemical staining of GFAP in surrounding astrocytes (3). Evidence exists that the colocalization of astrocytes with SP in the hippocampus occurs early in AD, often in the absence of dystrophic neurites suggesting that astrogliosis may not just be a response to neuronal injury but may also contribute to the AD process (3). In the neocortex, levels of GFAP messenger RNA (mRNA) have been reported to be correlated with SP density in temporal but not frontal neocortex in AD brains (4). Complicating these observations, animal research has shown that levels of GFAP and GFAP mRNA in brain tissue increase with age (5). Thus, it is necessary to consider the effects of aging when evaluating the association between GFAP levels in the brain and presence of AD.

Using a sandwich ELISA, we quantified GFAP levels without knowledge of diagnosis in four areas of neocortex in brains from individuals with AD, age-matched controls, and younger controls with

normal cognition prior to death. We speculated that not only would levels of GFAP be higher in the brains of AD subjects, but that the GFAP elevations would be highest in the neocortical region thought to be involved earliest in the AD process – the temporal lobe (6).

## Material and methods

### The study population

The Honolulu-Asia Aging Study (HAAS) began in 1991 as a supplement to the Honolulu Heart Program, a longitudinal study of cardiovascular disease in a cohort of Japanese-American men living on Oahu at the time of the baseline examination in 1965. The original cohort consisted of 8006 men born 1900 through 1919. Detailed descriptions of the study design have been previously published (7, 8).

### Dementia case-finding methods

Evaluation and follow-up for dementia began at the 1991–1993 examination of the cohort when the men were 71–93 years (average 78 years) of age and a second round of evaluations was carried out from 1994 to 1996. All participants received the Cognitive Abilities Screening Instrument (CASI) that has been validated and used in USA and Japan for evaluating cognitive function (9, 10). Scores ranged from 0 to 100 with 74 identified as the optimal score distinguishing demented from non-demented (11). Participants signed informed consent forms at each examination. The multi-step procedure used to identify cases of dementia has been described in detail elsewhere (9). Subjects suspected to have dementia based on poor performance on the CASI received a full diagnostic evaluation for dementia. This included a standardized interview and a neurological examination by a neurologist, as well as the neuropsychological test battery from the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) (12, 13). Those individuals judged by the study neurologist to meet *Diagnostic and Statistical Manual of Mental Disorders, Third Edition, Revised* (*DSM-III-R*) (14) criteria for dementia had brain computed tomographic scans and blood tests including complete blood count, chemistry profile, vitamin B12 level, red blood cell (RBC) folate level, rapid plasma reagin, and thyroid function test.

Final diagnosis and Clinical Dementia Rating index (15) were assigned by a clinical consensus committee that included the study neurologist and at least two other investigators.

Diagnosis of AD was made using the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria (16).

### Autopsy methods

A concentrated effort to obtain autopsies on all cohort deaths also began in 1991. The idea of autopsy was discussed with each participant. In the state of Hawaii the family must give final consent for autopsy after death. Research protocol autopsies are currently obtained on greater than 20% of all cohort deaths.

Brains were removed by a neuropathologist or neuropathology technician within 30 h of discovery of death (average time from death to autopsy was 14.6 h). Immediately following removal of the brain, tissue was resected (usually from the left hemisphere) for storage at −70°. For this study, frozen tissue from four brain regions: the frontal pole, temporal pole, superior lateral parietal cortex 2 cm posterior to the motor cortex, and the occipital pole were used. Gross examination of the brains was carried out by a neuropathologist (JH) who was blinded to the participant's clinical history. The microscopic examination included close inspection of sections of frontal, temporal, parietal, and occipital cortices, as well as sector CA1 and subiculum of the hippocampus stained with Hematoxylin and Eosin (H&E) and Bielschowsky stains and stains using antibodies directed against β-amyloid and ubiquitin. Microscopic sections were examined by one of the three study neuropathologists without knowledge of the clinical findings. Lesions including SP, neuritic plaques, and neurofibrillary tangles were counted per $mm^2$ (17) in five fields selected as having the most lesions for each of the four neocortical areas. The highest count among the five fields was taken to represent that area.

### Study design

For this case–control study, 10 AD cases meeting NINCDS-ADRDA clinical criteria for definite AD and meeting CERAD neuropathological criteria for definite or probable AD were selected from the HAAS autopsy archive. Ten age matched men without dementia were selected who scored 74 or higher on the CASI within 5 years of death (mean interval between CASI and death was 25 months) and who had less than two neuritic plaques per $mm^2$ in the

neocortex at autopsy. A third group of 10 younger controls were selected based on age less than 80 years at death, cognitive function score 74 or higher and having zero neuritic plaques in the neocortex at autopsy.

### GFAP assay and immunoblots

Frozen tissue blocks were removed from the storage tubes and held frozen on a cold plate. With the aid of a no. 11 scalpel, 25–50 mg of gray matter from each neocortical block were excised, weighed, and homogenized by sonification in 10 volumes of hot (90–95°C) 1% (w/v) sodium dodecyl sulfate (SDS).

Assays were performed without knowledge of case status or neuropathologic findings. Aliquots of the homogenates were assayed for GFAP using the sandwich ELISA of O'Callaghan (18). Briefly, a rabbit polyclonal antibody to GFAP (Dako Corp., Carpenteria, CA, USA) was coated on the wells of Immulon-2 microtiter plates (Dynatech Laboratories, Chantilly, VA, USA). After blocking non-specific binding with non-fat dry milk, aliquots of the homogenates were diluted in sample buffer and added to the wells of the plate. After appropriate blocking and washing steps, a mouse monoclonal antibody to GFAP (Chemicon, Temecula, CA, USA) was added to "sandwich" GFAP between the two antibodies. An alkaline phosphatase-linked antibody directed against mouse immunoglobulin G (IgG) (Dako Corp., Carpenteria, CA, USA) was then added, and a colored reaction product was obtained by subsequent addition of enzyme substrate. Quantification was achieved by spectrometry at 405 nm using a microplate reader (UV Max running on a Soft Max program, Molecular Devices, Menlo Park, CA, USA). Data are expressed as μg GFAP per mg total protein. This assay of GFAP has been cross-validated with another solid-phase immunoassay (18) and with densitometric analysis of Coomassie blue-stained GFAP resolved by two-dimensional electrophoresis (19).

GFAP immunoblots were performed to assess GFAP integrity in the same samples subjected to ELISA. Aliquots of the sample homogenates were resolved by SDS–polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA) and subjected to immunoblot analysis according to the procedure described by O'Callaghan (20). Detection of the immunoreactive bands was achieved using the [$^{125}$I] rProtein-A and the same monoclonal anti-GFAP antibody used in the GFAP ELISA.

### Statistical methods

Because of the small sample size within each of the three groups, non-parametric methods were used to describe and analyze the data. Comparisons between cases and age-matched controls for each region of the brain were made using the difference in GFAP levels between a case and an age-matched control based on the Wilcoxon signed rank test. These comparisons form an attempt to assess differences in GFAP levels within a region of the brain that can be attributed to AD and not to age. Within-region differences between AD cases and younger controls were also made, but here, significance testing relied on a Wilcoxon rank sum test (21).

**Table 1** Median levels of selected characteristics for the control groups and for the cases of Alzheimer's disease

| Characteristic | Controls | | Cases† |
|---|---|---|---|
| | Young* | Age-matched | |
| Age (years) | 77.0 (76–79)‡ | 83.5 (76–94) | 83.5 (76–92) |
| Education (years) | 12 (8–16) | 9.5 (6–20) | 8 (7–12) |
| Most recent CASI score | 85.3 (82.9–93) | 86.3 (73.5–93) | 4.7 (0–75.2) |
| Post-mortem interval (h) | 14.5 (4.1–23) | 13.3 (4.9–24.1) | 9.2 (6.1–30.6) |
| Maximum NFT count | 1.1 (0–2.7) | 0.5 (0–13.5) | 44.4 (0–99) |
| Maximum NP count | 0 (0–0) | 0 (0–1.5) | 9.7 (5.2–17) |
| GFAP (μg/mg total protein) | | | |
| Frontal | 10.0 (4.2–22.6) | 12.7 (1.5–27.6) | 10.5 (7.8–44.1) |
| Occipital | 5.8 (1.5–11.1) | 3.6 (0.7–9.4) | 11.9 (3.5–26.0) |
| Parietal | 8.3 (0.3–17.5) | 8.9 (3.3–23.3) | 14.7 (7.2–47.5) |
| Temporal | 12.3 (4.0–22.8) | 11.1 (2.9–41.4) | 39.7 (8.1–77.5) |

* Other than age, there were no significant differences between the two control groups for any of the other characteristics.

† Other than the selection criteria used to identify cases of Alzheimer's disease (most recent CASI score and maximum number of NP and NFT), there were no significant differences in any of the other characteristics between the cases of Alzheimer's disease and their age-matched controls except for levels of GFAP that were observed in the occipital, parietal, and temporal lobes ($P = 0.010$, $P = 0.028$ and $P = 0.004$, respectively).

‡ Range.

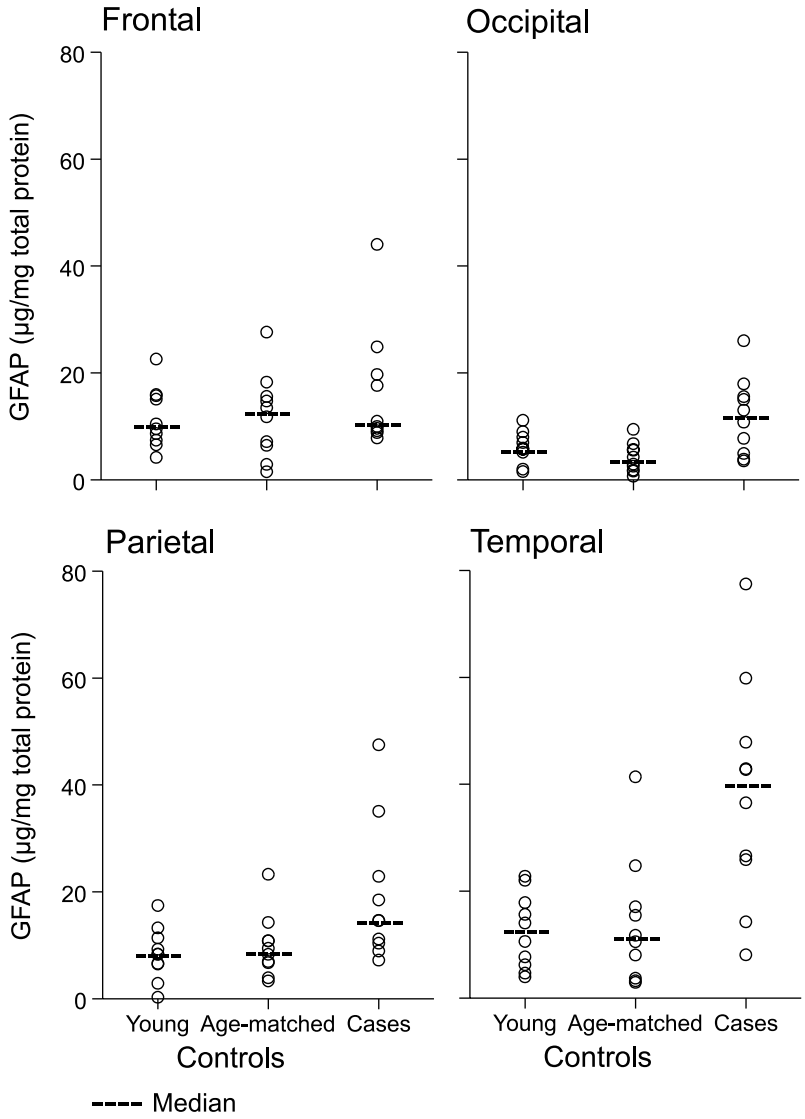


**Figure 1.** Individual GFAP levels in the two control groups (young and age-matched controls) and in the cases of Alzheimer's disease within each region of the brain.

## Results

Distributional characteristics of selection variables (last CASI score, maximum neurofibrillary tangle count, maximum neuritic plaque count) and the matching variable (age) confirm expected differences and similarities among the three groups (Table 1). The distribution of years of school attendance is highest among younger controls and lowest among cases of AD ($P = 0.08$). Although the median value of the post-mortem interval is notably smaller among cases of AD, the overall distributions of the interval among the three groups are indistinguishable ($P = 0.61$). There is no statistically significant association between post-mortem interval and GFAP levels in the control subjects (the lowest $P$-value for this relationship was in the temporal lobe; $P = 0.17$).

Frontal lobe GFAP levels are indistinguishable among the three groups (Fig. 1). However, for the remaining three regions (occipital, parietal, temporal), the GFAP levels are significantly higher for cases of AD vs their age-matched controls (Tables 1, $P = 0.010$, 0.028, 0.004, respectively). Furthermore, 80–90% of cases of AD have higher GFAP levels compared with their age-matched controls in occipital, parietal, and temporal regions (Fig. 2). Levels of GFAP are indistinguishable between the two control groups for all three of these brain regions. The highest measurements of GFAP were observed in the temporal region among cases of AD and the greatest GFAP excess in cases of AD vs age-matched controls was in the temporal

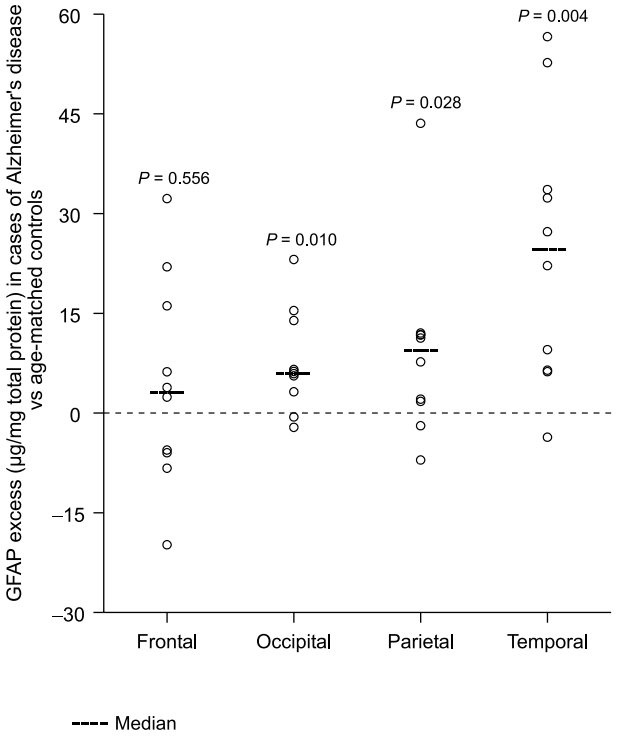


**Figure 2.** Excess individual GFAP levels in the cases of Alzheimer's disease as compared with their individually age-matched controls within each region of the brain. Reported *P*-values correspond to statistically significant excesses of GFAP levels in the cases of Alzheimer's disease.

lobe (Fig. 2). Results are essentially unchanged after removing from the analysis those subjects with a post-mortem interval greater than 18 h.

The GFAP immunoblot results provide additional confirmation for the ELISA method. A consistent band pattern was observed for all samples. A major band appears at approximately 50 kDa, consistent with the known molecular weight of GFAP (data not shown). Three to four minor immunoreactive bands are resolved below the major band corresponding to GFAP turnover products seen in experimental animals. For one of the 30 subjects, GFAP ELISA data for all brain regions are very low; immunoblots of these same samples reveal very low immunoreactivity and a loss of the 50 kDa band – findings indicative of proteolysis (post-mortem interval was 24.1 h). Exclusion of this individual from statistical analyses had no effect on findings.

## Discussion

Astrogliosis is a well known neuropathologic feature in AD and there is evidence supporting the association of reactive astrocytes with SP (22, 23). The temporal sequence of plaque formation, amyloid deposition, neuronal loss, and astrogliosis is not known; however, there is evidence that astrogliosis occurs early in AD perhaps in response to fibrillar Abeta deposits. This suggests that astrogliosis may contribute to the AD process (3).

To our knowledge, this is the only report of quantitative differences in GFAP levels across regions of the human brain that are associated with AD. Using an immunoassay procedure to quantify levels of GFAP, we confirmed that GFAP is elevated in the brains from subjects with AD and demonstrated that the greatest elevations are in the temporal lobe. The more than three-fold increase in temporal lobe GFAP found in this analysis of AD cases corresponds to a large degree of neural damage in this region.

While median GFAP levels were significantly higher in temporal, parietal, and occipital cortices of the AD brains compared with both control groups, no significant differences were found for the frontal cortex. These findings support another report (4) where GFAP mRNA levels were elevated in the temporal cortex of elderly women with AD and correlated positively with SP density while levels in the frontal lobe were not associated with SP density. It is notable that the regional pattern of elevated GFAP in the AD brains roughly corresponds to the marked hypometabolism of glucose predominantly involving the temporal and parietal lobes seen on positron emission tomography (PET) in patients with AD (24, 25). A similar pattern of neocortical hypometabolism has been seen in PET imaging of the non-human primate in response to a neurotoxic injury to the entorhinal cortex (26). In this study, there was a significant association between the degree of damage in the entorhinal cortex and the deficits in metabolism.

We observed no differences in the GFAP levels between the age-matched and young controls. This finding is counter to most reports in humans that show increased GFAP mRNA in the hippocampus, frontal and temporal cortex after the age of 60 in unaffected brains (27), and increases in GFAP in cerebrospinal fluid (CSF) with increasing age (28). Failure to find any difference may be because of limited age range and small sample size. Age related GFAP elevations might have already developed in our controls, the youngest of whom was 76 years.

Astrocytes are activated by proinflammatory mediators (cytokines, chemokines) expressed by activated microglia and macrophages. Neurotrophic factors [transforming growth factor-β (TGF-β)] and prostaglandins known to activate astrocytes are also elaborated by activated microglia (29). While astrocyte activation and subsequent gliosis has a putative role in healing following injury to the central nervous system, glial scarring may also have

a detrimental effect on neuronal function (30). This hypothesis may be especially relevant to the pathogenesis of AD. Colocalization of SP with reactive astrocytes but not dystrophic neurites supports the idea that astrogliosis may occur prior to and contribute to neuronal injury in AD (3). Inhibition of GFAP synthesis may delay astrogliosis and allow for axonal regeneration and remyelination. In the future, manipulation of factors that activate astrocytes may be an additional strategy for treating or preventing AD.


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## References


1. O'Callaghan JP, Miller DB, Reinhard JF Jr. Characterization of the origins of astrocyte response to injury using the dopaminergic neurotoxicant, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Brain Research 1990;**521**:73–80.
2. O'Callaghan JP. Quantitative features of reactive gliosis following toxicant-induced damage of the CNS. Ann N Y Acad Sci 1993;**679**:195–210.
3. Pike CJ, Cummings BJ, Cotman CW. Early association of reactive astrocytes with seniles plaques in Alzheimer's disease. Exp Neurol 1995;**132**:172–9.
4. Le Prince G, Delaere P, Fages C, Duyckaerts C, Hauw J-J, Tardy M. Alterations of glial fibrillary acidic protein mRNA level in the aging brain and in senile dementia of the Alzheimer type. Neuroscience Lett 1993;**151**:71–3.
5. Goss JR, Finch CE, Morgan DG. Age-related changes in glial fibrillary acidic protein mRNA in the mouse brain. Neurobiol Aging 1991;**12**:165–70.
6. Braak H, Braak E. Neuropathological staging of Alzheimer related changes. Acta Neuropathol 1991;**82**:239–59.
7. Kagan A. In: The honolulu heart program: an epidemiological study of coronary heart disease and stroke (Kagan A. eds.). Amsterdam: Hardwood Academic Publishers, 1996.
8. Syme SL, Marmot MU, Kato H, Rhoads G. Epidemiologic studies of coronary heart disease and stroke in Japanese men living in Japan, Hawaii and California: introduction. Am J Epidemiol 1975;**102**:477–80.
9. White L, Petrovitch H, Ross GW et al. Prevalence of dementia in older Japanese-American men in Hawaii: the Honolulu-Asia Aging Study. JAMA 1996;**276**:955–60.
10. Teng EL, Hasegawa K, Homma A et al. A practical test for cross-cultural epidemiological studies of dementia. Int Psychogeriatr 1994;**6**:45–58.
11. White L, Masaki K, Ross GW, Petrovitch H, Chiu D, Teng E. Estimation of the sensitivity and specificity of a dementia screening test in a population-based survey. Neurobiol Aging 1994;**15**(Suppl. 1):171.
12. Morris JC, Heyman A, Mohs RC et al. The Consortium to establish a registry for Alzheimer's disease (CERAD), part 1: clinical and neuropsychological assessment of Alzheimer's disease. Neurology 1989;**39**:1159–65.
13. Welsh K, Butters N, Hughes J, Mohs R, Heyman A. Detection of abnormal memory decline in mild cases of Alzheimer's disease using CERAD neuropsychological measures. Arch Neurol 1991;**48**:278–81.
14. American Psychiatric Association. Diagnostic and statistical manual of mental disorders, Revised 3rd edn. Washington, DC: American Psychiatric Association, 1987.
15. Berg L. Mild senile dementia of the Alzheimer's type: diagnostic criteria and natural history. Mt Sinai J Med 1988;**55**:87–96.
16. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease. Report of the NINCDS-ADRDA work group under the auspices of Department of Health and Human Services task force on Alzheimer's disease. Neurology 1984;**34**:939–44.
17. Petrovitch H, Nelson J, Snowdon D et al. Microscope field size and the neuropathologic criteria for Alzheimer's disease. Neurology 1997;**49**:1175–6.
18. O'Callaghan JP. Quantification of glial fibrillary acidic protein: comparison of slot-immunobinding assays with a novel sandwich ELISA. Neurotoxicol. Teratol. 1991; **13**:275–81.
19. Brock TO, O'Callaghan JP. Quantitative changes in the synaptic vesicle proteins synapsin I and p38 and the astrocyte-specific protein glial fibrillary acidic protein are associated with chemical-induced injury to the rat central nervous system. J Neurosci 1987;**7**:931–42.
20. O'Callaghan JP, Imai H, Miller DB, Minter A. Quantitative immunoblots of proteins resolved from brain homogenates: underestimation of specific protein concentration and of treatment effects. Anal Biochem 1999;**274**:18–26.
21. Rosner B. Fundamentals of biostatistics. Pacific Grove, CA: Duxbury, 2000.
22. Beach TG, Walker R, McGeer EG. Patterns of gliosis in Alzheimer's disease and aging cerebrum. Glia 1989;**2**:420–36.
23. Vijayan VK, Geddes JW, Anderson KJ, Chang-Chui H, Ellis WG, Cotman CW. Astrocyte hypertrophy in the Alzheimer's disease hippocampal formation. Exp Neurol 1991;**112**:72–8.
24. Kumar A, Schapiro MB, Grady C et al. Studies in Alzheimer's disease. Neuropsychopharmacology 1991;**4**: 35–46.
25. Benson DF. PET/dementia: an update. Neurobiol Aging 1988;**9**:87–8.
26. Meguro K, Blaizot X, Kondoh Y, Le Mestric C, Baron JC, Chavoix C. Neocortical and hippocampal glucose hypometabolism following neurotoxic lesions of the entorhinal and perirhinal cortices in the non-human primate as shown by PET. Implications for Alzheimer's disease. Brain 1999;**122**:1519–31.
27. Nichols NR, Day JR, Laping NJ, Johnson SA, Finch CE. GFAP mRNA increases with age in rat and human brain. Neurobiol Aging 1993;**14**:421–9.
28. Rosengren LE, Wikkelso C, Hagberg L. A sensitive ELISA for glial fibrillary acidic protein: application in CSF of adults. J Neuroscience Meth 1994;**51**:197–204.
29. Kreutzberg GW. Microglia: a sensor for pathological events in the CNS. Trends Neurosci 1996;**19**:312–8.
30. Eng LF, Lee YL. Intermediate filaments in astrocytes. In: Keffermann H, Ransom BR, eds. Neuroglia. Oxford: Oxford University Press, 1995: 650–67.